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RESEARCH COMMUNICATION

***p63* is the molecular switch for initiation of an epithelial stratification program**

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Development of stratified epithelia, such as the epidermis, requires *p63* expression. The *p63* gene encodes isoforms that contain (TA) or lack (Δ N) a transactivation domain. We demonstrate that TAp63 isoforms are the first to be expressed during embryogenesis and are required for initiation of epithelial stratification. In addition, TAp63 isoforms inhibit terminal differentiation, suggesting that TAp63 isoforms must be counterbalanced by Δ Np63 isoforms to allow cells to respond to signals required for maturation of embryonic epidermis. Our data demonstrate that *p63* plays a dual role: initiating epithelial stratification during development and maintaining proliferative potential of basal keratinocytes in mature epidermis.

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Development of the epidermis requires a series of coordinated events, which regulate proliferation and differentiation of keratinocytes. Although recent discoveries have highlighted a number of genes required for terminal differentiation of keratinocytes (Hu et al. 1999; Takeda et al. 1999; Rangarajan et al. 2001; Nickoloff et al. 2002; Nicolas et al. 2003), a question that remains is how the single-layered surface ectoderm commits to initiate stratification during embryogenesis. A candidate gene involved in this process is the transcription factor *p63*. *p63* is expressed in the surface ectoderm prior to stratification and continues to be expressed during embryonic development. As the epidermis matures, *p63* expression becomes restricted to the basal layer. In adult tissues, *p63* is expressed in stratified epithelia, whereas its expression is absent from single-layered epithelia (Yang et al. 1998). These expression data suggest a role for *p63* in the development and maintenance of stratified epithelia, such as the epidermis. In support of this hypothesis, investigators found that mice lacking *p63* display a fundamental defect in epithelial lineage development and fail to develop stratified epithelia and epithelial appendages, such as teeth, hair follicles and mammary glands (Mills

et al. 1999; Yang et al. 1999). The surface epithelium of *p63*^{-/-} newborn mice consists of a single cell layer that fails to express epidermal differentiation markers, including keratins K5 and K14 (Mills et al. 1999; Yang et al. 1999). The lack of stratification and the consequent absence of barrier formation in *p63*^{-/-} mice results in dehydration and death within hours after birth. This fundamental defect in stratified epithelial lineage development displayed by *p63*^{-/-} mice has led to two hypotheses concerning the potential role of *p63* in epidermal development. It has been proposed that *p63* may play a pivotal role in maintaining the stem cell population in an already committed stratified epithelium (Yang et al. 1999; Pellegrini et al. 2001). Alternatively, *p63* may play a critical role in the commitment to stratified epithelial lineages (Mills et al. 1999).

In an attempt to distinguish between these hypotheses, we further investigated the molecular role of *p63* in development of the embryonic epidermis and maintenance of the mature epidermis. *p63* is expressed in at least six isoforms (Yang et al. 1998). The use of alternative promoters and transcription start sites gives rise to two classes of *p63* transcripts: those encoding proteins with an amino terminal transactivation domain (TA isoforms) and those encoding proteins lacking this domain (Δ N isoforms). In addition, alternative splicing gives rise to three different carboxyl termini designated α , β , and γ . In vitro data suggest that Δ Np63 isoforms have a dominant-negative function toward TAp63 isoforms (Yang et al. 1998); however, Δ Np63 isoforms were also shown to be able to transactivate target gene expression (Dohn et al. 2001; King et al. 2003; Wu et al. 2003), possibly because of the presence of a second transactivation domain within Δ Np63 isoforms (Ghioni et al. 2002). It has been reported that the predominant *p63* isoform expressed in mature epidermis is Δ Np63 α (Yang et al. 1998; Liefer et al. 2000).

We now demonstrate that TAp63 isoforms are the first *p63* isoforms expressed during embryogenesis and that they are required for initiation of epithelial stratification. Furthermore, ectopic expression of a TAp63 isoform in a simple epithelium in vivo resulted in the induction of squamous metaplasia, confirming the role of *p63* as a master molecular switch. We also show that TAp63 isoforms inhibit terminal differentiation, suggesting that TAp63 isoforms must be counterbalanced by Δ Np63 isoforms to allow cells to respond to signals required for maturation of embryonic epidermis. Our data are consistent with *p63* playing a dual role: initiating epithelial stratification during development and maintaining proliferative potential of basal keratinocytes in mature epidermis.

Results and Discussion

From the expression pattern of *p63* and the phenotype of *p63*^{-/-} mice, we hypothesized that *p63* is required for the induction of an epithelial stratification program. In support of this hypothesis, we have isolated *p63*^{-/-} cells from the surface epithelium of E18.5 *p63*^{-/-} mice. Like *p63*^{-/-} skin, *p63*^{-/-} cells failed to express K5 and K14 (Fig. 1), differentiation markers that are expressed in epithelia that have committed to initiate a stratification program (Byrne et al. 1994), as well as terminal differentiation markers K1 and loricrin (data not shown). Instead, *p63*^{-/-}

[**Keywords:** *p63*; stratified epithelia; molecular switch; embryogenesis; terminal differentiation; proliferative potential]

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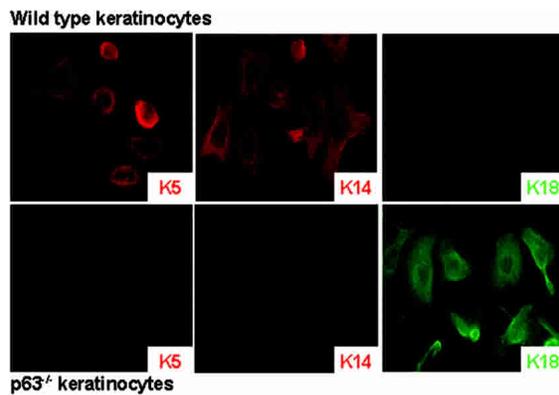


Figure 1. Primary $p63^{-/-}$ surface epithelial cells are blocked in their commitment to a stratified epithelial lineage. Differentiation markers K5 and K14, which are expressed in epithelia that have committed to a stratification program, are not expressed in primary $p63^{-/-}$ cells. These cells do, however, express K18, a marker for single-layered epithelia.

cells expressed K18, which is normally synthesized in the surface ectoderm prior to stratification and in adult single-layered epithelia (Jackson et al. 1981; Moll et al. 1982). These data demonstrate that without $p63$, surface epithelial cells are intrinsically blocked in their commitment to become a keratinocyte.

To study the role of $p63$ during epidermal morphogenesis, we determined the expression profiles of TA- and $\Delta Np63$ isoforms during embryonic development. We found that $\Delta Np63$ isoforms were first expressed at E9.5, after the developing epidermis has committed to stratification, but prior to terminal differentiation (Fig. 2a; Byrne et al. 1994). However, TAp63 isoforms were detected as early as E7.5, prior to the commitment to stratification. In addition, we observed nuclear p63 staining in every cell in the surface ectoderm of E8.5 embryos, suggesting that expression of TAp63 isoforms is required in this tissue (Fig. 2b,c). Based on these data, we hypothesized that TAp63 isoforms may be required for the initiation of an epithelial stratification program. To test this hypothesis, we transfected expression constructs encoding TAp63 α , TAp63 γ , $\Delta Np63\alpha$, and $\Delta Np63\gamma$ into cells that do not express p63 or K14 (Ptk2 cells, HR9 cells, and F9 cells) and analyzed the induction of K14 expression. As expected from their expression patterns, we found that only TAp63 isoforms were able to induce K14 expression, whereas $\Delta Np63$ isoforms did not affect K14 expression (Fig. 3a). To determine whether TAp63 isoforms could initiate a stratification program in vivo, we ectopically expressed TAp63 α in single-layered lung epithelia by using “gene-switch” mice (Wang et al. 1997, 1999; Cao et al. 2002). The regulator of the gene-switch system (Glp65) is activated by progesterone antagonists such as RU486 and was placed under control of the surfactant protein C promoter (SP-C.Glp65), as previously described (Zhao et al. 2001). The SP-C promoter targets transgene expression to the cuboidal, surfactant-secreting type II alveolar cells and to the distal Clara cells, which line the bronchioles of the lung (Wikenheiser and Whitsell 1997). Neither of these cell types normally expresses $p63$ (Yang et al. 1998) or markers of stratified epithelial differentiation, K5 and K14 (Fig. 3e,f; Kasper et al. 1993). Adult bigenic SP-C.Glp65/TAp63 α mice, SP-C.Glp65/ $\Delta Np63\alpha$ mice, and control mice were treated

with 500 $\mu\text{g}/\text{kg}$ RU486 once daily for 7 d, which resulted in induction of transgene expression (Fig. 3b). Lung biopsy specimens demonstrated that ectopic TAp63 α expression resulted in the induction of K5 and K14 expression in type II alveolar cells (data not shown). Furthermore, squamous metaplastic lesions developed in the bronchioles (Fig. 3d), which expressed K5 and K14 (Fig. 3e; data not shown). Similar results were obtained when E18.5 lungs were analyzed from embryos in which TAp63 α expression was induced starting at E8.5 by injecting pregnant mice daily with 100 $\mu\text{g}/\text{kg}$ RU486 (Fig. 3f; data not shown). In contrast, ectopic expression of $\Delta Np63\alpha$ using the same gene-switch system did not alter lung morphology and did not result in induction of K5 and K14 (Fig. 3c; data not shown). Taken together, these data demonstrate that TAp63 α , but not $\Delta Np63\alpha$, can commit a single-layered epithelium to initiate a stratification program.

To further analyze the role of $p63$, we used the gene-switch mice to target the overexpression of TAp63 α to the basal layer of the epidermis and hair follicles by using a K14 promoter (K14.Glp65; Berton et al. 2000; Cao et al. 2002). Adult (Fig. 4a–c) or newborn (data not shown) mice were treated topically once daily for 5 d with 100 μg RU486 to induce TAp63 α expression. Skin biopsy specimens exhibited severe hyperplasia (Fig. 4a), and BrdU incorporation assays demonstrated that this hyperplasia was the result of hyperproliferation (Fig. 4b). In control epidermis, BrdU-labeled cells were detected sporadically in the basal layer. However, induction of TAp63 α expression resulted in an increase in BrdU-labeled cells in the basal layer as well as the presence of BrdU-labeled cells in the suprabasal layers of the epidermis (control 4.6 ± 2.1 cells/mm vs. TAp63 α -induced 97.2 ± 5.0 cells/mm, $P < 0.01$; Fig. 4b). In addition, induction of TAp63 α expression in the epidermis resulted in

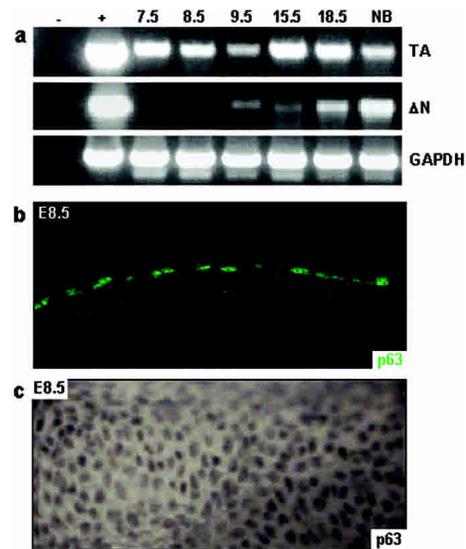


Figure 2. Differential expression of p63 isoforms occurs during embryonic development. RT-PCR analysis using primers specific for TA- or $\Delta Np63$ demonstrated that TAp63 isoforms are expressed as early as E7.5, whereas $\Delta Np63$ isoforms are not expressed until E9.5 (a). Immunofluorescence (b) and whole-mount immunohistochemistry (c) using the mAb4A4 antibody confirmed nuclear localization of TAp63 isoforms in the surface ectoderm of E8.5 embryos.

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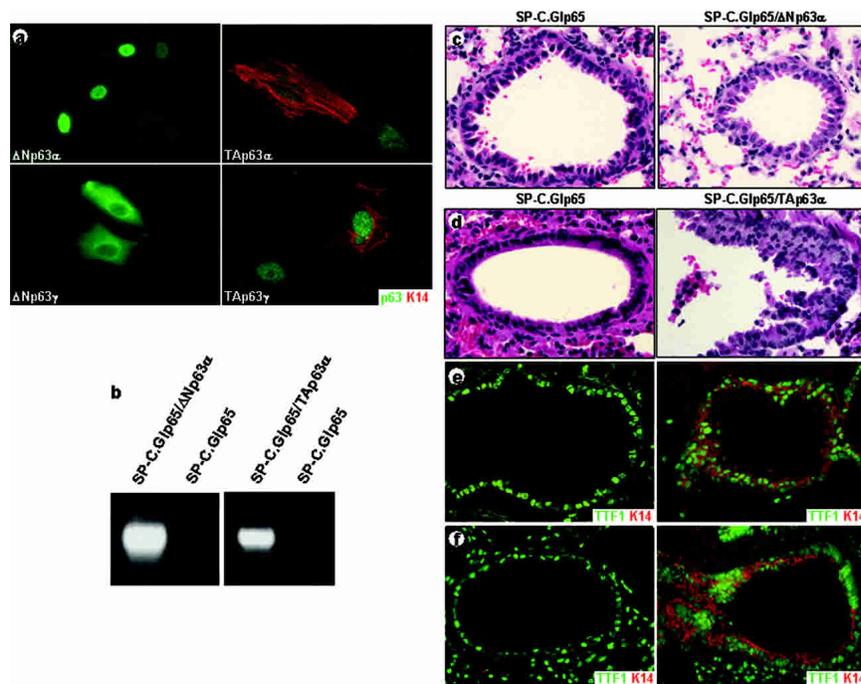


Figure 3. Ectopic expression of TAp63 α initiates a stratification program in vitro and in vivo. Immunofluorescence on Ptk2 cells transfected with the indicated p63 expression constructs demonstrated that only TAp63 isoforms could induce K14 expression (~75% of the transfected cells; *a*). To determine if p63 could also induce a stratification program in vivo, we induced ectopic expression of TAp63 α or Δ Np63 α in single-layered lung epithelia by using gene-switch mice. Induction of TAp63 α or Δ Np63 α transgene expression was determined by RT-PCR (*b*). Ectopic expression of Δ Np63 α in single-layered lung epithelia did not alter lung morphology (*c*). Ectopic expression of TAp63 α , however, resulted in squamous metaplastic lesions in the bronchioles (*d*) and induction of K14 expression, a marker for stratified epithelia, in adult (*e*) and E18.5 (*f*) lungs. TTF1 staining was used to identify type II alveolar cells and distal Clara cells in the bronchioles.

perturbed epidermal differentiation as demonstrated by a delayed onset of K1 expression (Fig. 4c), indicating an apparent failure of the basal keratinocytes to commit to terminal differentiation. We also induced TAp63 α expression in developing embryos starting at E8.5. Skin biopsies taken at E18.5 demonstrated that induction of

TAp63 α expression in embryonic epidermis, like that in newborn and adult epidermis, resulted in severe hyperplasia (Fig. 4d), hyperproliferation (data not shown), and a delayed onset of differentiation (Fig. 4e).

In an attempt to determine if TAp63 α expression could completely block terminal differentiation, we initiated RU486 treatment at E3.5 and analyzed the skin phenotype at E13.5. At this stage, in control embryos, the epidermis covering the fore- and hindlimbs had initiated stratification and had formed the future spinous layer, which expressed K1 (Fig. 4f). However, induction of TAp63 α expression in embryonic epidermis resulted in a failure to initiate stratification (Fig. 4f). To confirm that TAp63 α expression prevents terminal differentiation, we isolated primary keratinocytes from the epidermis of newborn K14.Glp65/TAp63 α mice and control mice. When primary keratinocytes are maintained in media with a low Ca²⁺ concentration (0.05 mM), they proliferate and do not undergo terminal differentiation. When the Ca²⁺ concentration is increased (0.1 mM), primary keratinocytes differentiate and initiate expression of K1 (Fig. 4g; Yuspa et al. 1989). However, when we induced TAp63 α expression in primary keratinocytes, the cells failed to differentiate after increasing the Ca²⁺ concentration and did not initiate K1 expression (Fig. 4h). In addition, it was

recently demonstrated that overexpression of Δ Np63 α in primary mouse keratinocytes prevents Ca²⁺-induced differentiation (King et al. 2003). Taken together, these findings support an additional role for p63 in maintaining keratinocytes in the proliferative compartment and in preventing their entry into terminal differentiation.

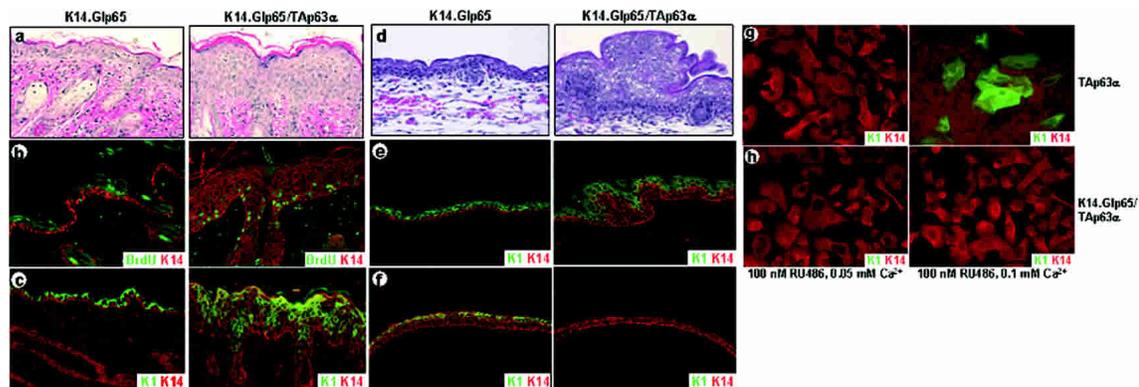


Figure 4. Induction of TAp63 α expression results in hyperplasia, hyperproliferation, and inhibition of terminal differentiation. Induction of TAp63 α expression in adult skin resulted in hyperplasia (*a*), hyperproliferation as demonstrated by a BrdU incorporation assay (*b*), and a delayed onset of terminal differentiation as demonstrated by a delayed onset of K1 expression (*c*). Induction of TAp63 α at E8.5 resulted in hyperplasia (*d*) and a delayed onset of K1 expression (*e*) at E18.5. When TAp63 α expression was induced starting at E3.5, at E13.5 the developing epidermis covering the fore- and hindlimbs demonstrated a lack of K1 expression (*f*). The ability of TAp63 α to block terminal differentiation was further demonstrated by the failure of primary keratinocytes, in which TAp63 α expression was induced, to undergo Ca²⁺-induced differentiation (*g,h*).

On the basis of our data, we hypothesize that one role of $\Delta Np63\alpha$ during epidermal development is to counterbalance the effects of TAp63 α on terminal differentiation, thereby allowing cells to respond to terminal differentiation signals. To test whether this is true in vivo, we mated the components of the gene-switch system into the $p63^{+/-}$ background, in which expression levels of all p63 isoforms are 50% of the levels in wild-type mice. Because $\Delta Np63\alpha$ is the predominant p63 isoform expressed in the mature epidermis (Yang et al. 1998; Liefer et al. 2000), this decrease in p63 isoform expression mostly affects $\Delta Np63\alpha$ levels. TAp63 α expression was induced in a $p63^{+/-}$ background in newborn (Fig. 5a–d) or adult (data not shown) mice. The shift in p63 isoform expression results in a situation where TAp63 isoforms are the predominantly expressed isoforms, thereby mimicking the $p63$ expression profile during early epidermal development. After 4 d of daily topical treatments with 100 μ g RU486, newborn K14.Glp65/TAp63 $\alpha/p63^{+/-}$ mice appeared dehydrated and their skin exhibited cracks and peeling (Fig. 5a). Immunofluorescence analysis demonstrated that K1 expression was confined to the outermost layers of the epidermis where the skin was intact (Fig. 5c), and loricrin expression was completely absent (Fig. 5d). The lack of loricrin expression would impair formation of epidermal barrier function and result in water loss (Koch et al. 2000). In addition, an examination of adjacent eroded areas revealed that the epidermis was completely missing, suggesting that the marked inhibition of terminal differentiation also resulted in epidermal fragility (Fig. 5b–d). In essence, this phenotype resembles the immature embryonic skin at E14.5–E15.5, just prior to epidermal stratification. In summary, these data demonstrate that induction of TAp63 α in a $p63^{+/-}$ background results in an increased severity of the inhibition of terminal differentiation. Because in vitro data demonstrated that $\Delta Np63\alpha$ has a dominant-negative function toward TAp63 α (Yang et al. 1998), we speculate that the switch in expression of p63 isoforms toward $\Delta Np63$ isoforms during epidermal development is required to counterbalance the inhibitory effect of TAp63 α on terminal differentiation.

On the basis of our data, we propose that TAp63 α is the molecular switch that initiates an epithelial stratification program. Execution of this program requires a shift in the balance between the p63 isoforms toward $\Delta Np63$ isoforms to allow keratinocytes to respond to signals required for maturation of the epidermis. Once the mature epidermis is formed, persistently elevated p63 levels in the basal layer of the mature epidermis are required for maintaining the proliferative potential of keratinocytes. The proliferative capacity of basal keratinocytes may be maintained through inhibition of p21 and 14-3-3 σ expression via direct binding of $\Delta Np63\alpha$ to their promoters (Westfall et al. 2003). Withdrawal from the cell cycle and commitment to terminal differentiation may be partially orchestrated by signaling through Notch and IKK α , two molecules required for terminal differentiation of the epidermis (Hu et al. 1999; Takeda et al. 1999; Rangarajan et al. 2001; Nickoloff et al. 2002; Nicolas et al. 2003).

Materials and methods

Primary keratinocyte culture

$p63^{-/-}$ cells were isolated from the surface epithelium of E18.5 $p63^{-/-}$ mice. Skins were floated overnight on DispaseII (Roche) at 4°C and pri-

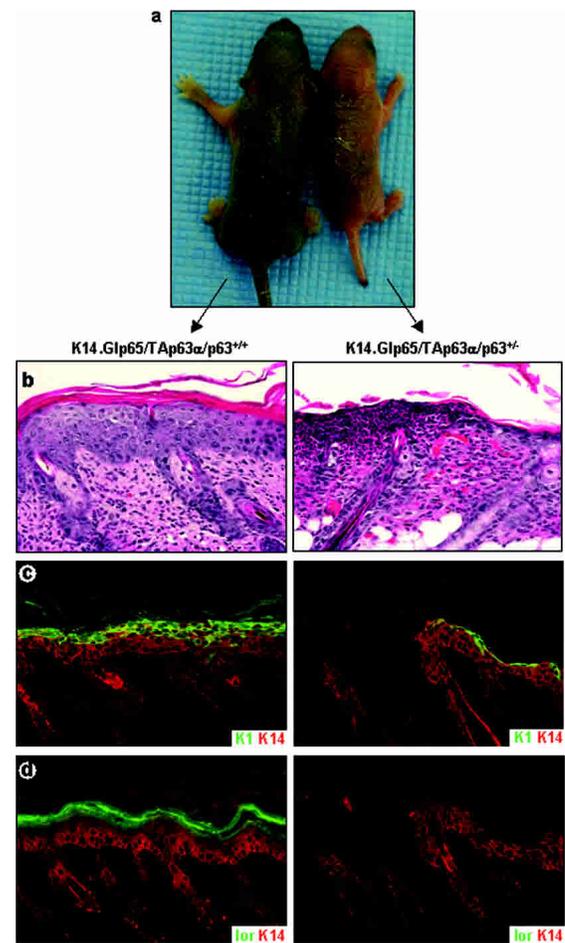


Figure 5. Induction of TAp63 α expression in the presence of reduced levels of $\Delta Np63\alpha$ results in epidermal fragility. TAp63 α expression was induced in the epidermis of K14.Glp65/TAp63 α mice on a $p63^{+/-}$ or $p63^{+/+}$ genetic background. Although K14.Glp65/TAp63 $\alpha/p63^{+/+}$ mice looked macroscopically normal after 4 d of RU486 treatment, K14.Glp65/TAp63 $\alpha/p63^{+/-}$ mice developed fissures in the epidermis (a). Histologically, the lesions in K14.Glp65/TAp63 $\alpha/p63^{+/-}$ epidermis appeared eroded (b–d). In the epidermis adjacent to these lesions, K1 expression was confined to the outermost layers of the epidermis (c), and loricrin expression was absent (d), providing an explanation for the apparent fragility of the epidermis.

mary keratinocytes were isolated as described previously (Yuspa et al. 1989). Cells were cultured in 50% fibroblast-conditioned medium supplemented with 0.05 mM Ca²⁺ and 4 ng/mL epidermal growth factor. Immunofluorescence was performed 48 h after plating. Cells were subcultured when the cultures reached 90% confluency.

For the in vitro differentiation assays, primary keratinocytes were isolated from the epidermis of newborn K14.Glp65/TAp63 α and control mice (K14.Glp65 and TAp63 α monogenic mice) as described previously (Yuspa et al. 1989). Transgene expression was induced by supplementing the media with 100 nM RU486. For the differentiation assays, the Ca²⁺ concentration in the media was increased to 0.1 mM 7 d after plating the cells, and cells were harvested for immunofluorescence 24 h later.

Cell lines and transfections

Cell lines that do not express p63 and K14 were identified using RT-PCR and immunofluorescence. Ptk2 cells (rat kangaroo kidney epithelial cells) were grown in supplemented EMEM medium (Cambrex Bio Science). HR9 cells (mouse embryonic endodermal cells) and F9 cells (mouse embryonic teratocarcinoma cells) were grown in supplemented DMEM medium (Invitrogen). Cells were transfected with expression constructs

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encoding myc epitope-tagged TAp63 α , Δ Np63 α , TAp63 γ , and Δ Np63 γ (Yang et al. 1999) using FuGene6 (Roche). Cells were harvested for immunofluorescence 48 h posttransfection.

Transgenic/knockout mouse lines

To generate the inducible TAp63 α / Δ Np63 α transgenes, we cloned the cDNAs encoding mouse TAp63 α / Δ Np63 α into the UAS-TK vector or UAS-TATA vector, respectively (Wang et al. 1997). Transgenic mice were generated by standard techniques. Founder mice were identified by tail tip DNA PCR analysis using primers TK.FW (5'-GGTCGAAAGCG GAGTACTGTC-3') and m63-2R (5'-GCATCGTTTCACACCTCG-3'). K14.Glp65, SP-C.Glp65, and p63^{+/-} mice were previously generated and characterized (Mills et al. 1999; Zhao et al. 2001; Cao et al. 2002).

Administration of RU486 to mice

To induce transgene expression in SP-C.Glp65/TAp63 α and SP-C.Glp65/ Δ Np63 α mice, we injected 500 μ g/kg RU486 (Mifepristone; Biomol), dissolved in sesame oil, i.p. once daily for 7 d. Adult or newborn K14.Glp65/TAp63 α and control mice were treated topically for 5 d with 100 μ g RU486, dissolved in 70% ethanol. To induce transgene expression in utero, we injected pregnant mice daily with 100 μ g/kg RU486 and 0.5 mg progesterone (to prevent potential abortion; Sigma), dissolved in sesame oil. Transgene induction in bigenic mice was confirmed by RT-PCR and RNase protection assays.

In vivo BrdU incorporation and analysis

Newborn or adult mice were injected i.p. with 250 μ g/g BrdU (Sigma) in 0.9% sterile saline solution. Skin biopsies were taken 1 h later and fixed in 10% neutral buffered formalin (NBF). To determine the proliferative index, we counted four fields of cells from four independent samples and calculated the average number of BrdU positive cells per mm skin.

Immunofluorescence and whole-mount immunohistochemistry

Lung tissue was fixed in 10% NBF by gravity perfusion through the cannulated trachea. Skin biopsies were fixed flat in 10% NBF. Cultured cells were fixed in cold methanol. Primary antibodies used for immunofluorescence were guinea pig anti-K14 (Yuspa et al. 1989), rabbit anti-K5 (Yuspa et al. 1989), rabbit anti-K6 (Mills et al. 1999), rabbit anti-K1 (Yuspa et al. 1989), rabbit anti-loricrin (Mills et al. 1999), mouse anti-K18 (Sigma), TTF-1 (thyroid transcription factor 1), mAb4A4 (p63; Yang et al. 1999), and FITC-conjugated anti-BrdU (Becton Dickinson). Secondary antibody conjugates used were Alexa-conjugated fluorochromes 594 goat anti-guinea pig, 488 goat anti-rabbit, 594 goat anti-rabbit, and 488 goat anti-mouse (Molecular Probes). For whole-mount immunohistochemistry, embryos were fixed in 4% paraformaldehyde. Embryos were incubated with the mAb4A4 antibody followed by biotinylated horse anti-mouse (Vector Laboratories) and ABC Elite reagent (Vector Laboratories). To detect the substrate, we incubated embryos in DAB/NiCl₂ followed by H₂O₂.

RT-PCR analysis

To determine the expression profile of p63 isoforms during embryogenesis, we obtained timed pregnant ICR mice from Harlan. RNA was extracted using RNeasy Kits (Qiagen) from whole embryos for stages E7.5 through E9.5 and from epidermis for stages E15.5 through newborn. Reverse transcription of 2 μ g of RNA was performed using random hexamers (Promega) and Moloney murine leukaemia virus reverse transcriptase (Promega). TAp63, Δ Np63, and GAPDH expression were detected by RT-PCR using primers m63TA.F (5'-TCGCAGAGCAC CCAGACA-3') and m63-2R for TAp63; m63 Δ N.F (5'-TTGTACCTG GAAAACAATG-3') and m63-2R for Δ Np63; and GAPDH1 (5'-AAGGT CGGTGTGAACGGATT-3') and GAPDH2 (5'-TGGTGGTGCAGGAT GCATTG-3') for GAPDH. To confirm transgene induction in gene-switch mice, we detected TAp63 α and Δ Np63 α transgenes by RT-PCR using primers TK.FW and m63-2R.

All experiments involving mice were performed under IACUC approval (Protocol number: AN-546).

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